OPTIMIZATION OF *ASPERGILLUS TUBINGENSIS* GROWTH FOR CELLULASE PRODUCTION

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ABSTRACT: Celluloses are the basic component of plant cell walls which are difficult to break however, cellulase enzymes have the ability to degrade the cellulose into glucose. The use of celullase enzymes is spreading widely particularly in the paper, pulp and food industries to reduce biowaste. The purpose of this study was to optimize the production of cellulase enzymes by using fungal strain *Aspergillus tubingensis*. The fungal strain *Aspergillus tubingensis* was extracted from putrid fruits and vegetable samples collected from the local fruit market of Pattoki in the District Kasur. The identification of fungus was carried out using microscope. Corn Stover was used as a substrate for cellulase production. Different parameters were used for the optimization of fungus growth such as substrate level, substrate particle size, temperature, pH, moisture, urea and glucose. Cellulase enzymes were purified by salting out and gel filtration techniques. The optimum substrate level, substrate particle size, temperature, pH, glucose, urea and moisture were analyzed as 5g, 40mm, 40°C, 4.5pH, 0.5g, 0.04g and 60% respectively. The maximum activity reveled by cellulase enzyme at different optimized parameters were reported in U/mL/min. The cellulase molecular weight was determined 71 kDa by Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis. It was concluded that the study will help to a way for the scientists for the maximum production of cellulase from *Aspergillus tubingensis* using Corn stover as substrate and most cost-effective Solid State Fermentation method.

Key words: *Aspergillus Tubingensis*, Cellulose, Cellulase, Substrate, Optimization, Solid State Fermentation.

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INTRODUCTION

Cellulases are the enzymes capable of hydrolyzing the cellulose into smaller sugar components. Cellulolytic enzyme is complex of enzymes such as exoglucanase, endoglucanase, and beta glucosidase which plays an important role in the natural biodegradation of lignocellulosic materials (Lynd *et al.*, 2002). The Cellulase enzymes degrade the ß-1,4-glycosidic linkage of cellulose and convert them in to glucose monomers thus overcoming the problems of their complex structure (Gao *et al.,* 2008; Kim *et al.,* 2008; Imran *et al.,* 2017).

These enzymes can be synthesized by the microorganisms on different cellulose containing substrates (Koo, 2001). Filamentous fungi also have the potential of utilizing cellulosic material as the source of carbon and energy and produce cellulase enzyme (Alriksson *et al.,* 2009). In the cellulase production mechanism fungi are used their elongated hyphae to exert mechanical pressure on the cellulose structure and as a result inflicting fungi produced massive supply of cellulase (Sanche, 2009; Idris *et al.,* 2017). Production of cellulases from various fungi and bacteria (*Penicillium spp.*, *Aspergillus spp.*, Trichoderma, *Pseudomonas fluorescens*, *Bacillus subtilis*, *E. coli*, and *Serratia marscens*) for industrial use is in practice (Henriksson *et al.,* 1999; de Lima *et al*., 2005; Imran *et al*., 2011; Sethi *et al*., 2013; Gunny *et al*., 2015; Imran *et al*., 2016; Imran *et al*., 2018; Imran *et al*., 2018). Different types of defined substrates can be used for fermentation however, agro-industrial wastes are also most often used due to their abundant availability and recycling purpose (Acharya *et al*., 2008; Lu *et al*., 2010; Gautam *et al*., 2011). Agricultural waste such as rice straw, coffee husk, corn cobs and corn Stover are also used as substrate (Milala *et al*., 2005; Sanche, 2009). Cellulases are most attributed and third most widely used industrial enzymes. Different methods are in practice for cellulase production and Solid state fermentation (SSF) is one of the most commonly used method for its production. SSF is cost effective and time saving method as reported by Brijwani and Vadlani, (2011). About 3.5 billion tons of Cellulase is produced annually by the solid state fermentation method. The advantage of exploitation of SSF is to get lowest cost fermentation system and likelihood to use it at industrial scale (Graminha *et al*., 2008). These enzymes are used in processing of starch, fermentation units, malting fruits and vegetables, juices extraction, ethanol production, animal food additives, paper recycling and textile industry (Gao *et al*., 2008; Ljungdahl, 2008; Zhou *et al*., 2008; Sukumaran *et al*.,

2009; Onofre *et al*., 2013). Although, there is always a need to find more efficient fungal strains that produce cellulases in order to meet the demands of industrial uses and can work in an easy and cost effective manner. The present study aimed to find optimized conditions for the growth of *Aspergillus tubingensis* cellulase enzyme using SSF method.

MATERIAL AND METHODS

Collection of samples: Samples of putrid fruits and vegetables were collected from the local fruit market of Pattoki, Kasur district, Pakistan.

Isolation and identification of fungal species: Samples collected from different locations were cultured on Potato dextrose agar, and kept for 3–7 days at 30 °C. Pure colonies of fungus were obtained by streak plate method. Fungal strains were identified based on morphological characteristics and spore staining (Alsohaili and Bani-Hasan, 2018).

Inoculum preparation: After identification, inoculums were prepared using the spores of *Aspergillus tubingensis*. For inoculum preparation a 500 mL flask distilled water (100 mL), glucose (2.0g/L), Ammonium sulphate (0.02g/L), Calcium chloride (0.05g/L) Magnesium Sulphate (0.05g/L) Potassium di-hydrogen phosphate (0.2g/L) were added. Flask was kept on a continuous shaker at 120 rpm and 30°C until a milky white color was obtained. This solution was used for further experimental work so stored at 4 °C.

Substrate preparation: Samples of Corn Stover were collected and dried in the hot air oven for 48 hours. Then, samples were ground and stored in airtight glass jar. The samples were further analyzed for approximate analysis (starch, fats, protein, and Cellulose). The approximate analysis was performed using Infra-Red Spectroscopy in Animal Nutrition Laboratory in Central Laboratory Complex, Ravi campus Pattoki, Pakistan.

DNS Reagent preparation: Di nitro salicylic acid (DSA) was prepared using principles of Imran *et al*. (2017). All the ingredients were mixed with the help of a hot water bath at 80°C till a clear crystal solution was obtained and kept in a brown bottle to prevent photo oxidation.

Effect of substrate: Corn Stover was used as the substrate and its five levels (3, 4, 5, 6 and 7 g) were used for the optimization of fungus. One gram was kept as a control. For this 5 mL inoculum was added in each flask and kept at room temperature for 3 days. After 3 days 100 mL acetate buffer (pH 4.5) was added, centrifuged at 5000 rpm for 5 minutes. One mL supernatant was taken with one mL DNS and 0.1 M mL cellulose solution. The spectrophotometer was used at 540 nm to assess the absorbance and cellulase activity was obtained for each sample. All samples were treated in triplicates.

Effect of substrate particle size: Different substrate particle sizes were used as 30, 40, 50, 60, and 70mm while 10mm was used as control. To see the difference of substrate particle size was repeated as reported by Imran *et al*. (2017).

Effect of temperature: To optimize the fungus growth five different temperatures such as 30, 40, 50, 60, and 70 °C were used while, 37°C was kept as control. Five-gram substrate (Corn stover) was mixed with 5 mL inoculum in a flask. Flask was kept at different temperatures for 3 days and the procedure was repeated as reported by Imran *et al*. (2017).

Effect of Moisture and pH: Moisture and pH has a great effect on the growth of fungus. For the optimization of fungus growth different pH parameters from pH 3-7 and moisture 30- 70 % were set with 1pH and 3 and 0% control respectively. To see the effect of moisture and pH methodology of Imran *et al*. (2017) was followed.

Effect of glucose and urea: Different levels of glucose (0.3, 0.4, 0.5, 0.6, 0.7 g) and urea (0.01, 0.02, 0.03, 0.04 and 0.05 g) were used. To see effect of glucose and urea methodology of Imran *et al*. (2017) was followed.

Purification of enzyme and Gel Filtration Chromatography: Purification of cellulase enzyme was carried out by salting-out method and dialysis tube method. In salting out method different concentrations of ammonium sulfate (30-80%) were made and precipitated enzyme was separated by using dialysis tube in this different buffers of 3-10 pH ionic strength were used. For further purification gel filtration chromatography was performed with Sephadex G-100 and purified enzyme using methods of Bokhari *et al*. (2009). The cellulase elusions were collected from 1-20 and their activity was checked by the proposed procedure given by Iqbal *et al*. (2011).

SDS PAGE: SDS PAGE was used to determine the molecular weight of the cellulase enzyme by following the methods of Coral *et al*. (2002), and Dutta *et al*. (2008).

Statistical Analysis: The collected data were subjected for descriptive statistics and Analysis of variance (ANOVA) by using Statistical Package for Social Sciences (SPSS) (Version 21.0).

RESULTS AND DISCUSSION

In the present study *Aspergillus tubingensis* were identified on the basis of morphological and spore staining characteristics using a fluorescent microscope. Optimization of fungus was carried out on different

parameters like substrate levels, substrate particle size, pH, temperature, urea and glucose. This study is important for cellulase production and to provide information about *Aspergillus tubingensis* and its optimization. For the maximum production of cellulase different substrate levels (3-7g) were used. The best optimized level was recorded as 5g with activity 8.09±0.6 U/mL/min (Figure 1). It was noticed that as the quantity of the substrate increased the activity of cellulase also increased gradually. The recorded activity of cellulase at 3g and 5 g substrate levels were 5.51±0.30 U/mL/min and 8.09±0.6 U/mL/min respectively but after 5g till 7 g (5.31±0.30) a decrease in cellulase activity was noticed. The cellulase activity was recorded by using spectrophotometer absorbance of 540 nm with one mL DNS solution and one mL (0.1 M) cellulose solution. Moreover, 3.24±0.30 U/mL/min activity was recorded with control. All the treatments were analyzed by ANOVA and significantly different from the control ($p<0.05$). The gradual decrease and increase in the result is due to fungus growth. The production of cellulase is directly proportional to the fungus growth as the fungus growth increased or decreased cellulase production was effected. The optimized substrate for fungal growth was noticed as 5g and low as compared to findings by Imran *et al*. (2017). However, the cellulase activity results were comparable and 5g with the substrate (Liang *et al*., 2012).

Five levels were set for the optimization of temperature for the fungal growth. It was noticed 40°C was the optimized temperature with the maximum activity of 18.03±.20 U/mL/min (Figure 2). Other parameters (30, 50, 60 and 70°C) which were set showed with the increase of temperature Cellulase activity was increased but after the optimized level (40°C) the activity decreased. Activity at 37°C was recorded as17.52±.17 U/mL/min. The activity of Cellulase was affected due to fungal growth, fungus growth is effected from a high range of temperatures because high temperature not favorable for fungus (Shabeb *et al*., 2010). In the previous study optimized temperature was 25 °C, however, at this temperature the cellulase production was less as compared to current study for industrial scale hyper production of cellulase is required so 40°C give a higher quantity of cellulase (Nitin, 2012).

Six different pH ranges (pH 3-7) were used for fungus growth. The different pH levels were maintained by acetate buffer. In the present study the optimized pH was noticed as 4.5 with an activity of 29.6±.48 U/mL/min (Figure 3). It was noticed that enzyme activity was increased gradually from pH 3 to pH 4 and highest at pH 4.5 but further increase (pH 5-7) in pH reduced the enzyme activity. A minimum activity was noticed on pH 7. While, some other studies reported 7.0 pH as optimum pH for the fungus growth and Cellulase production (Panagiotou *et al*., 2003). Similarly, a study was conducted in 2012 according to that study optimum pH was 6.9 (Kaushal *et al*., 2012). Comparable to our findings El-Nahrawy, (2017) affirmed that 5.0 pH was recorded for *Aspergillus tubingensis* to show maximum cellulase production with SSF. In the current study, the optimum pH level was recorded less as compared to previous studies. The higher pH level affects the fungus growth and also denatures the Cellulase enzyme.

Latifian, (2011) additionally underscored the significance of moisture level for the most extreme organism development and cellulase creation by directing analysis by two species of *Trichoderma reesai* for cellulase generation under SSF. The moisture level 40, 55, and 70% were utilized and most extreme action was found on 70% where as in ongoing investigation the greatest cellulase movement was found on 60% with activity 17.46±.13 U/mL/min as shown in (Figure 4). Therefore, the ideal moisture content 60% is critical because it is truly appropriate for strong state maturation on modern scale. However, Imran *et al*. (2017) reported 50 % moisture level was optimum with the same fungal strain but in Response Surface Methodology (RSM) which was less as compared to our existing findings. Similarly Liang et al. (2012) reported only 30 % moisture content with *Aspergillus* spp. and rice grass as a substrate using RSM. While, Sun *et al*. (2010) and Oberoi *et al.* (2014) also provided higher moisture contents of 70 and 72% appropriate for the trichoderma and *Aspergillus spp.*, respectively to produce cellulase enzyme in SSF conditions. In one more study efficacy of cellulase was even recorded at 89.5% moisture content while using SSF and wheat straw as a substrate (Pensupa *et al*., 2013).

Some other organic salts were also used for the hyper production of cellulases like glucose and urea. Five different quantity levels of glucose (0.3 to 0.7g) were used. The maximum activity was obtained at 0.5 glucose level and maximum activity at this level was 26.80±.60 U/mL/min (Figure 5). A minimal change in enzyme activity was recorded with different levels of glucose (0.3 and 0.4 g) and study showed a minor increase (0.6 and 0.7g) in glucose level may decrease the enzyme activity. So, precise glucose levels may be very important to determine the enzyme activity level.

Same as different urea levels (0.01 to 0.05) were used and the maximum activity was noticed at 0.04g of urea with 33.46±.30 U/mL/min activity (Figure 6). It was noticed in the present study levels of urea along with glucose were very critical in enzyme activity as 0.04 g was recorded for maximum activity of the enzyme but further enhancement in this dose such as 0.05 g reduced the activity. Imran *et al*. (2017) reported maximum cellulase activity was recorded with 0.3g of urea. Soni *et al*. (2010) also suggested that the production of cellulase was enhanced by using urea (0.01 to 0.05) because it acts as nitrogen source and fungus strain has specific compatibility with a nitrogen source. In recent work, urea

was also used as a nitrogen source to enhance the production of cellulase.

The salting out method is the best and easy method. Different concentrations of ammonium sulfate were made and add 20mL of crude cellulase in each concentration then kept for 30 min at room temperature. The activity was checked by spectrophotometer. It was noticed 70 % concentration of ammonium sulfate showed the maximum activity of 45.4±.21 U/mL/min of Cellulase (Figure 7). The precipitated salt was separated by dialysis tube method. For the dialysis tube method, different pH solutions were made at 1-10 pH. The crude cellulase was filled in dialysis tube with each pH and kept overnight. The further purification was carried out in gel filtration twenty elusions were collected and activity was assessed that showed eleven fractions had the maximum absorbance selected for further purification. The crude activity was increased by gel filtration and maximum activity was recorded with elusion 11 as 56.79 U/mL/min (Table 1).

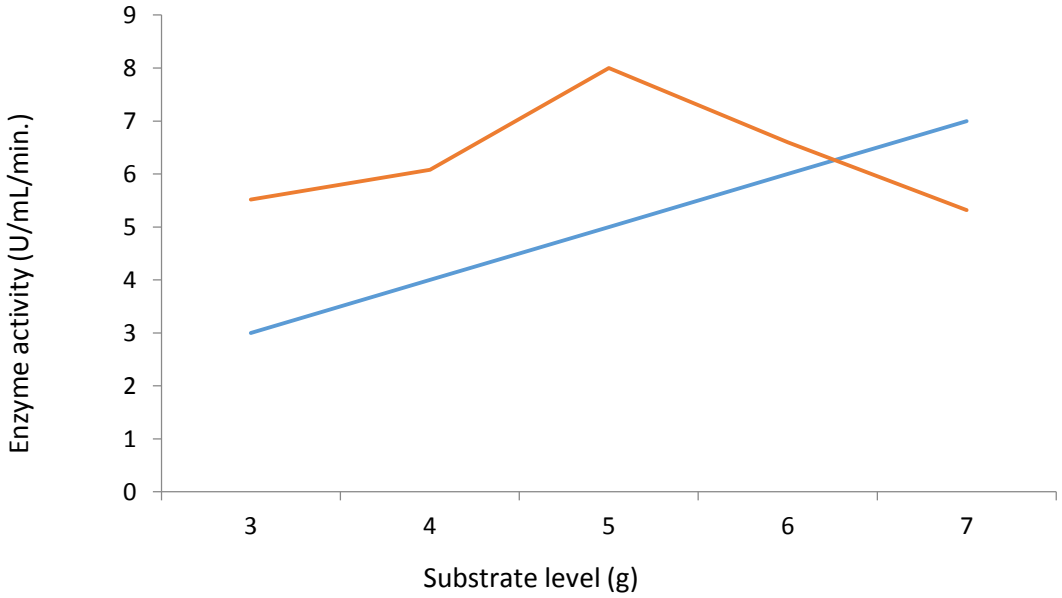

Figure 1. Effect of substrate level on the fungus growth

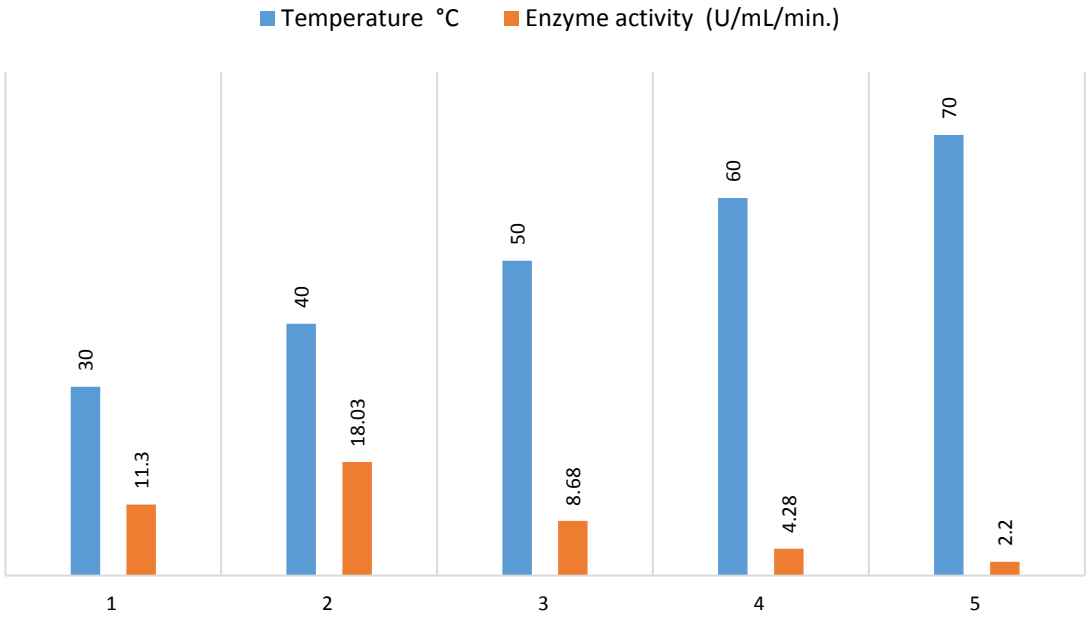

Figure 2. Effect of different temperature levels on fungus growth

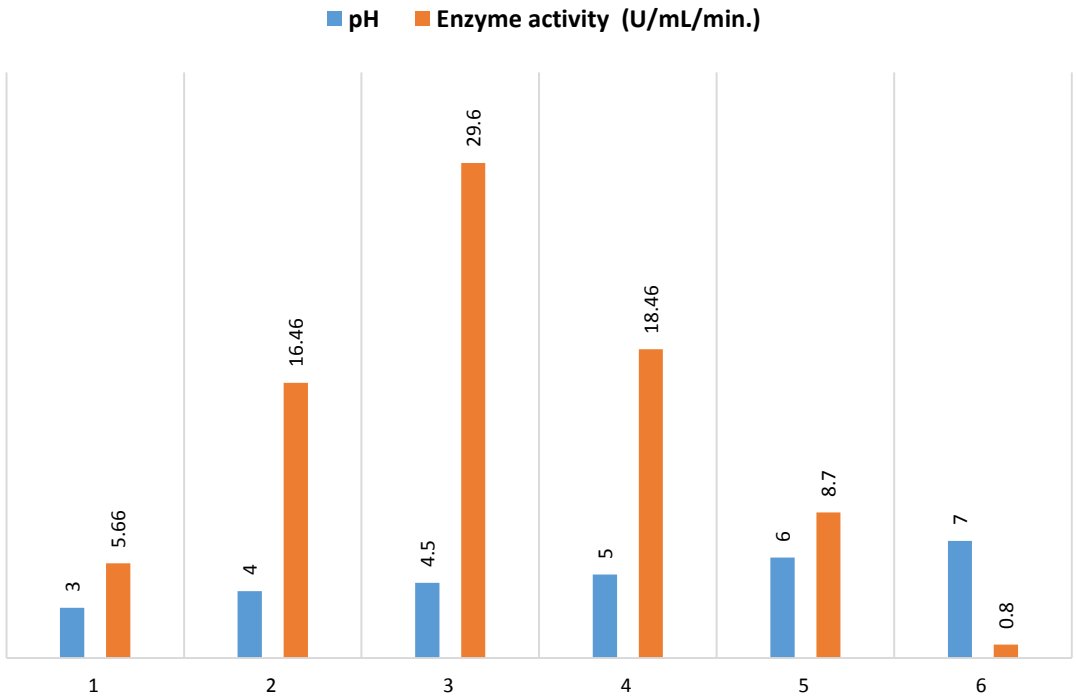

Figure 3. Effect of different pH levels on fungus growth

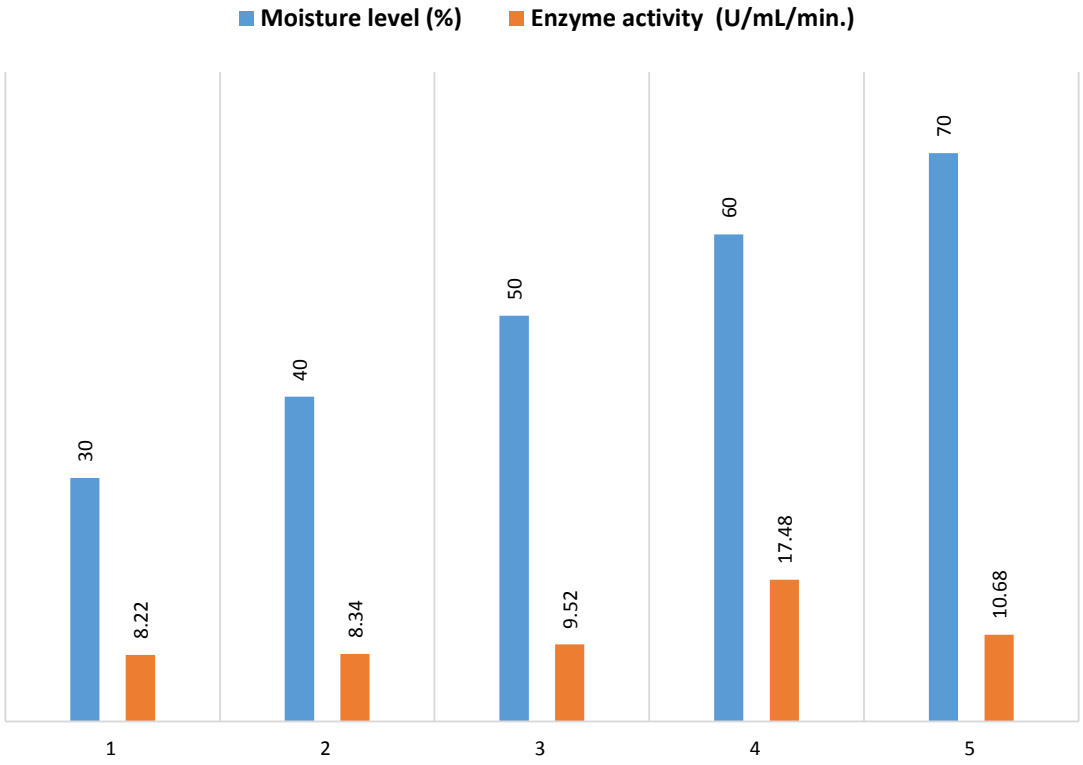

Figure 4. Effect of different Moisture levels on fungus growth

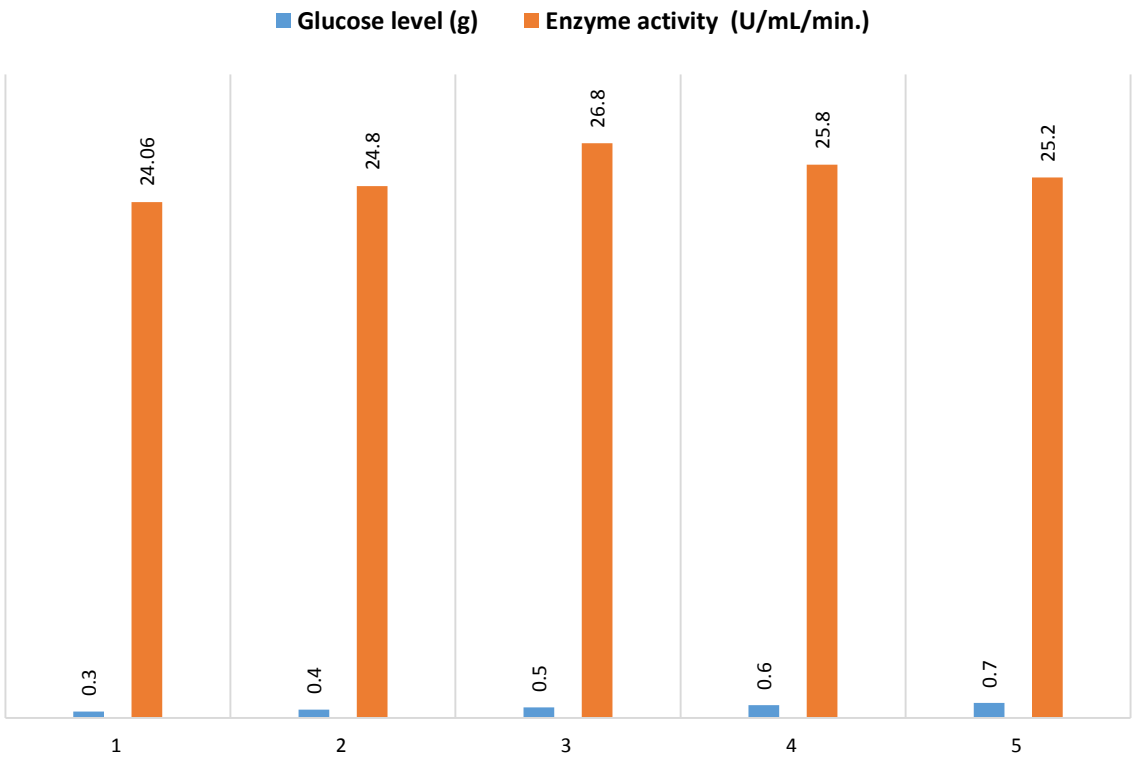

Figure 5. Effect of different glucose levels on fungus growth

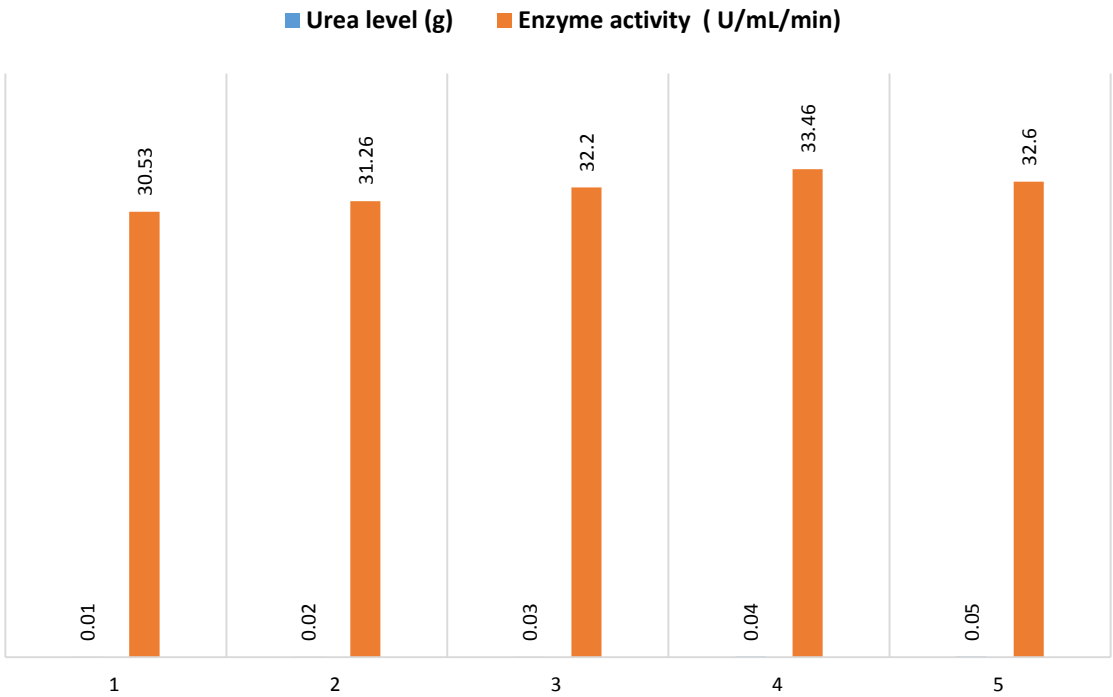

Figure 6. Effect of different urea levels on fungus growth

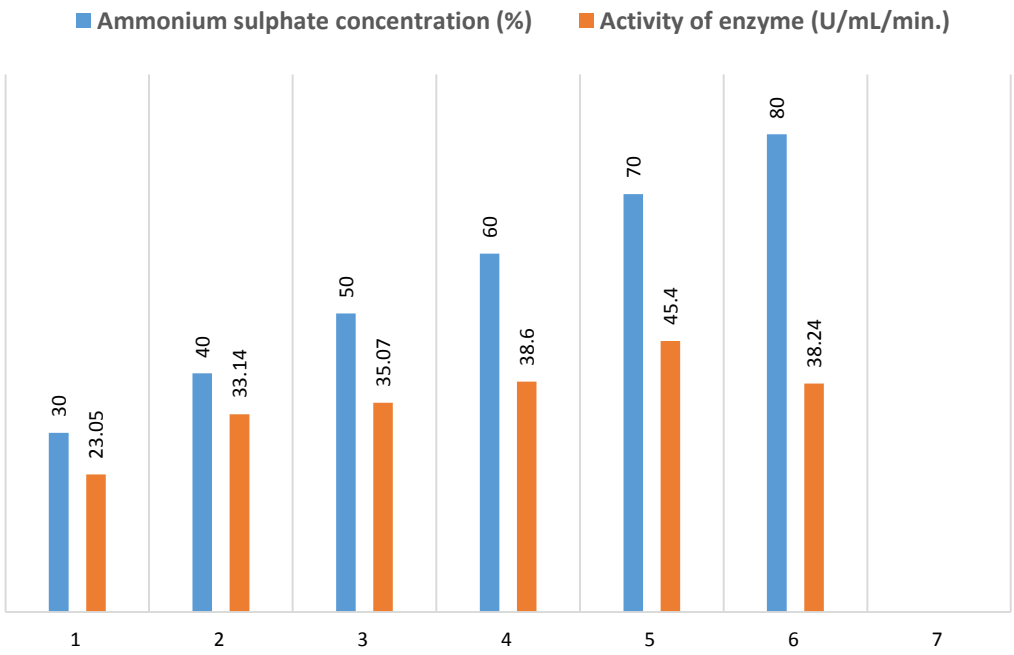

Figure 7. Cellulase activity at different concentration of ammonium sulfate

Table 1. Cellulase Enzyme Activity at different Elusions of gel filtration.

Elusion	Activity (U/mL/min)
1	24.21
2	24.25
3	25.02
4	25.35
5	35.38
6	35.68
7	36.91
8	53.12
9	53.51
10	56.71
11	56.79
12	56.64
13	55.34
14	54.31
15	54.18
16	54.12
17	54.08
18	54.06
19	53.98
20	53.65

Conclusion: The study provided the most efficient fungal strain which may be proven as efficient producers of cellulase enzyme at the industrial level. It is also informative as optimized conditions were obtained to provide standards against various parameters. The putrid vegetables and fruit samples proved to be a good source of fungal (*Aspergillus tubingensis*) isolation. Further studies for the solid-state fermentation can be fruitful in selecting *Aspergillus tubingensis* as the best strain for high level production of cellulase.

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